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Virulence and Immunogenicity of Typhoid Microbe Cultures, Immunogenicity of the Corresponding Vaccines and the Content of Vi-antigen in Them as Determined by the Hemagglutination Reaction.

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Since the time of the discovery of the typhoid microbe's superficial Vi-antigen (Velike, 1934), a great quantity of works related to the investigation of this antigen's characteristics has been published. However, the comparative complexity of the method, imposed by the presence of another somatic antigen (O-antigen), did not permit the complete discovery of this antigen's importance in the virulence and immunogenicity of the microbe.

In 1951 Spaun proposed to use a method of erythrocytic agglutination for the separate investigation of these two antigens. The method was based on the fact that the Vi-antigen dissolves readily in water and is quickly adsorbed onto erythrocytes (the erythrocytes are thereby sensitized), whereas the O-antigen remains connected with the bacterial cell. The erythrocytes start to agglutinate with a serum containing Vi-antibodies. These data were corroborated by Korvaz'e (1952), Minor, Minor and Grabar (1952), Kozinskii and coworkers (1952-1954), Lendi and coworkers (1952, 1953) and Khuber (1953 and other years). * (See Translators Note No 1)

However, there were no attempts in these works to compare the characteristics shown by a hemagglutination reaction, to the basic characteristics of the microbe cell. Therefore, we undertook the task of filling this gap as far as possible.

The first stage of our work was the determination of the optimum conditions of the hemagglutination reaction. We investigated the capacity of Vi-antigen-containing extracts, produced by different methods, to adsorb to the erythrocytes of different types of animals. The influences of the temperature, the concentration of hydrogen ions and of the adsorption time on the outcome of the hemagglutination reaction were also investigated. As a result, we selected the optimum and most easily fulfilled conditions for the staging of the hemagglutination reaction, as shown in the following chart.

1. The process of adsorption of the Vi-antigen to the erythrocytes.
 - a. Antigen - Supernatant Liquor of a suspension of living culture or vaccines.
 - b. Erythrocytes - Chicken, 10 % suspension in the supernatant liquor of the cultures or vaccines.
 - c. Adsorption temperature - Room temperature.
 - d. Adsorption time - 10-20 minutes.
2. The arrangement of the hemagglutination reaction.
 - a. Serum - Rabbit, containing Vi-antibodies diluted two-fold, in a 0.2 ml volume per dilution.

- b. Erythrocytes - Sensitized, not washed from the antigen, a 0.5-1 % suspension in 0.2-0.5 ml volume to every serum dilution.
- c. Glassware - Special plates or test tubes.
- d. Temperature of the reaction - Room temperature.
- e. Reaction Time - 30-40 minutes.
- f. Calculation of results - By the four-plus system.

However, the use of such a method did not permit a quantitative determination of the Vi-antigen content in the liquors under investigation, as minimum quantities of the antigen were required in the solution for the sensitization of the erythrocytes, and the reaction in all cases attained the titer of the Vi-antibodies in the serum. Therefore, we developed a method for the quantitative determination of Vi-antigen that allowed us to compare the extracts by their Vi-antigen content. It was based on the determination of the highest dilution of the extract capable (with the adsorption on the erythrocytes) of producing agglutination of the erythrocytes in the presence of the immune serum.

In table 1 are shown the results of the quantitative determination of the Vi-antigen in the extract procured by the Spaun method. It is apparent that the extract dilution of 1:80, selected for the sensitization of the erythrocytes, insured erythrocytic agglutination up to the titer of the serum. Therefore, the dilution, 1:80, may be accepted as the titer of the Vi-antigen in the extract. The hemagglutination method was utilized by us for the comparison of the virulence and immunogenicity of the vaccines to the quantitative content of Vi-antigen in them.

However, such a comparison can only be produced with the observance of fixed conditions. First, the suspension from which the supernatant liquors are prepared must contain the very same quantity of microbes. Second, the time intervals from the moment of washing of the culture and from the drawing of the supernatant liquors must be invariable. Therefore, for the determination of Vi-antigen, we always used suspensions containing one milliard microbes in one ml. It was shown by special tests that holding the suspension at room temperature for one hour was sufficient for the transfer of the essential quantity of the Vi-antigen from the microbic cell to the surrounding medium. Therefore, the method of receiving the supernatant liquor for the sensitization of the erythrocytes (to be used in the investigation of the Vi-antigen in living cultures) was as follows: We washed off a growth of agar culture with a physiologic solution. The specification of the suspension was set at one milliard living microbes in one (1) ml. The suspensions were left at room temperature for one (1) hour. After this, the centrifugation was completed. Then, the supernatant liquors were drawn off for the determination of their Vi-antigen content.

Fourteen (14) strains of typhoid fever cultures were investigated. Of these, two (2) strains (H-901 and O-901) did not sensitize the erythrocytes, five (5) strains (Nos 7655, 7705, 799, 30 and 62) sensitized the erythrocytes only with the use of the undiluted supernatant liquor, one (1) strain (No 18) sensitized the erythrocytes in a 1:2 dilution, two (2) strains (Nos 7675 and Betnagar) sensitized the erythrocytes in a 1:4 dilution and four (4) strains (Ti-1, Nos 1203, 208 and Ty₂⁴⁴⁴⁶) sensitized the erythrocytes in a 1:8 dilution.

The virulence of all these strains had been verified by tests on mice. The mice were intraperitoneally inoculated with a 24 hour culture of a strain in various doses which permitted the determination of the LD₅₀ of every strain. The death of the mice were taken into account for 72 hours. All necessary calculations were conducted by the Reed and Munch* (See Translator's note No 2) method.

From table No 2 it is apparent that the decrease of the LD₅₀, as a rule, accompanied an increase of the Vi-antigen titer. The strains not containing Vi-antigen or containing the latter in low titers (1:4) belonged correspondingly to the strains of little or medium virulence. The strains containing Vi-antigen in a greater quantity (1:8) belonged to the more virulent group.

The following series of experiments included the determination of the typhoid fever microbic strains' immunogenicity in mice and the comparison the data received, with the quantitative content of Vi-antigen in the corresponding vaccines. With this purpose, the 24 hour growth of an agar culture of the strains under investigation was washed off with a physiologic solution and the resultant suspension heated at 56° for one (1) hour. After an appropriate control on the sterility of the resultant vaccines, they were tested on mice and by the hemagglutination reaction.

The immunogenicity of the heated cultures were verified in the following manner. Each vaccine, in 100 million microbic bodies amounts, was twice subcutaneously injected into a group of sixteen (16) mice with a week's interval between the injections. On the tenth day after the completion of the vaccination, all of the mice were intraperitoneally inoculated with a virulent culture of Ty₂₄₄₄₆. Each group (16) of mice was divided into four (4) subgroups (4 in each group) and inoculated with $\frac{1}{2}$, 1, 2 and 4 Dcl respectively (1 Dcl was first tested for three (3) days and equalled 75 million living microbes). The mice of the control group were inoculated simultaneously with lesser experimental doses of the culture ($\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ and 1 Dcl). The death of the mice was observed over a 72 hour period after which the results were processed by the Reed and Munch formula for the determination of the LD₅₀. The test was repeated twice, and the corresponding average of the LD₅₀ was computed.

The hemagglutination reaction was arranged by the usual method. Varied dilutions of the heated cultures' supernatant liquor were used for the sensitization of the erythrocytes. It is evident from table 3 that should an injection of over 100 million microbes of the virulent culture produce a 50 % death rate in a group of mice that had been immunized by the heated cultures containing Vi-antigen at a 1:16 titer, then, the same effect would be achieved by an injection of only 25 million microbes for a group of mice that had been immunized by the heated cultures not containing Vi-antigen. An intensification of the heated cultures' immunogenic properties is derived by increasing the Vi-antigen content in them.

The investigation concerning the dependence of the immunogenicity upon the quantitative content of Vi-antigen in the produced formovaccines was of significant interest. The determination of Vi-antigen in vaccines by

using a reaction with an antimicrobic Vi-serum is practically impossible because the majority of the vaccines do not agglutinate with Vi-serum, and if they do agglutinate, they do so only to a slight degree. Therefore, the hemagglutination reaction was utilized with an application of the diluted supernatant liquors of the vaccines.

By this method we investigated 21 series of tetravaccine prepared in various production institutes of the USSR, one series of formolvaccine prepared from the Ty₂⁴⁴⁴⁶ strain, and two series of the polyvaccine NIISI. It is of interest to note that the latter did not sensitize the erythrocytes and the hemagglutination reaction with it was negative. The most probable explanation of this is that the typhoid fever antigen located in the vaccine was apparently already bound by an adsorbent. The test of all the listed vaccines showed that the highest dilutions of the supernatant liquor capable of sensitizing the erythrocytes varied within the different vaccines from 1:4 to 1:32.

The immunogenicity was checked in 18 series of the tetravaccines. The method of immunization and inoculation of the mice was the same as with the investigation of the heated cultures. The only difference was that in this test the mice were inoculated with three (3) doses (1, 2 and 4 Dcl) of a virulent Ty₂⁴⁴⁴⁶ culture.

As the single-momental* (See Translator's note No 3) checking of the many vaccines did not seem possible, we included one and the same vaccine in every series as a standard for the comparison of the results received from the various series of tests. We adopted the tetravaccine of series No 24, which was prepared in the Gamaleya Institute of the Academy of Medical Science of the USSR, as that standard. In all, four (4) series of tests were conducted. For the comparison of all the results in the different series, we took, as a basis, the data received in the series in which the effectiveness index of the standard vaccine was the lowest. For each of the remaining series of tests, we found the number which showed how many times the effectiveness index of the vaccine in series No 24 was larger than the index in the series of the test taken as a basis. Into this number were divided the effectiveness indices of each of the vaccines entering the given series of the test.

It is evident in table No 4 that there was no strict dependence between the amount of the effectiveness index, speaking of the immunogenicity of the vaccine, and the quantity of the Vi-antigen determined by the agglutination reaction. However, if the Vi-antigen titer was small (1:4), the immunogenicity of the vaccine was low. And the majority of the vaccines containing Vi-antigen at a 1:8 titer belonged to the vaccines with a lower immunogenicity than the vaccines having a Vi-antigen titer of 1:16.

CONCLUSIONS

1. The use of the hemagglutination reaction, belonging as do all serological tests to the supplementary methods of Vi-antigen investigation, enabled us to present the following advantages of this method:

- a. The absence of the non-specific reactions associated with the presence of other antigens and antibodies (O-antigen and O-antibodies).
- b. The possibility of the determination of the quantitative volume of Vi-antigen in the preparation under investigation.
- c. The possibility to determine the antigen in a dissolved state.

2. A study of the cultures' virulence compared to the quantitative volume of antigen in them(as determined by the hemagglutination reaction) allowed us to determine that with an increase of Vi-antigen in the cultures, their virulence was increased.

3. The immunogenicity of the warmed vaccine was in direct dependence on the quantitative volume of Vi-antigen in the supernatant liquor obtained by centrifuging the vaccine.

4. The immunogenicity of the formolic tetravaccines depended to a known degree upon the quantitative volume of Vi-antigen in it. However, a complete conformity between the quantity of Vi-antigen and the immunogenic properties of the vaccine did not develop in all cases.

Translator's notes:

1. Names are transliterated from the Russian text, and in actuality could be spelled differently.
2. Same as No 1.
3. The Russian word "odnomomentno" is unfamiliar to the translator. It could mean "instantaneously" or perhaps "simultaneously".

Table No. 1.
Determination of the Vi-antigen titer in the test extract.

The erythrocytes processed by the extracts in the injection	Dilution of the extract			Seroologic Solution
	1:40	1:80	1:160	
1:20	++++	++++	++++	-
1:40	++++	++++	++++	-
1:80	++++	++++	++++	-
1:160	++	+	-	-
1:320	-	-	-	-
Non-sensitized erythrocytes (Control)	-	-	-	-

Table No. 2.
Virulence of the cultures and their Vi-antigen content.

Strain	LD ₅₀ of the culture in millions of liv- ing microbes.	Vi-antigen titer by the homologous reaction.
0-901	700.7	None
11-901	420	None
7655	353	1
7705	323	1
789	270	1
30	237	1
62	237	1
7675	149.5	1:4
13	140	1:2
Botnagar	125	1:4
71-1	105	1:8
2000	75	1:8
205	75	1:8
1-2	75	1:2

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Table No 3
Immunogenicity of the cultures and their Vi-antigen content.

Strain	The LD ₅₀ determined in the tests			The Vi-antigen titer by the hemagglutination reaction
	1st	2nd	Average	
695	11.8	37.5	24.6	-
H-901	26.5	18.75	26.5	-
7655	59.5	37.5	48.5	1:4
30	53	53	53	1:4
799	67.2	58	62.6	1:4
Betnagar	75	66.8	70.9	1:8
7675	94.5	53	73.7	1:8
58	94.5	59.4	76.9	1:8
208	119	66.8	92.9	1:16
18	106	106	106	1:4
1203	104	118.5	111.2	1:16
Ty ₂ ⁴⁴⁴⁶	127	106	116.7	1:16
Vi-I	150	150	150	1:16
Non-immun- ized Mice (Control)	18.75	11.1	14.9	

Table No 4

Immunogenicity of the vaccines expressed in indices of effectiveness. Also, the Vi-antigen titers in the vaccines.

Vaccine		Index of effectiveness	Vi-antigen titer by the re-vaccination of hemagglutination.*
City	Series No		
Gor'kii	62	1.19	1:4
Khar'kov	89	1.49	1:16
Tbilisi	60	1.53	1:4
Khabarovsk	18	1.66	1:8
Kazan'	41	1.66	1:8
Pern'	45	1.76	1:8
Moscow	42	1.72	1:8
Khar'kov	82	1.84	1:16
Gor'kii	54	1.85	1:32
Kazan'	33	1.88	1:16
Irkutsk	36	1.88	1:16
Leningrad	105	2.06	1:16
Odesa	5	2.2	1:16
Moscow	35	2.22	1:8
Leningrad	28	2.33	1:16
Moscow	24	2.65	1:8
Leningrad	121	2.8	1:16
Khar'kov	96	3.1	1:16

*Translators note-In the translator's opinion, the heading of the fourth column should read as follows:

"The Vi-antigen titer as determined by the hemagglutination reaction".